Cloning of Codon Optimized Subtilisin E in pTEF-MF

Introduction

As the test expression of the Subtilisin E gene from *Bacillus subtilis* was not successful, we used a codon optimized variant of the Subtilisin E gene. As a host we chose the *S. cerevisiae* strand CENPK2-1D again as it has the deficiencies we need for selection. For vector backbone we tried the constitutive pTEF-MF vector again. A scheme of the cloning can be seen below:

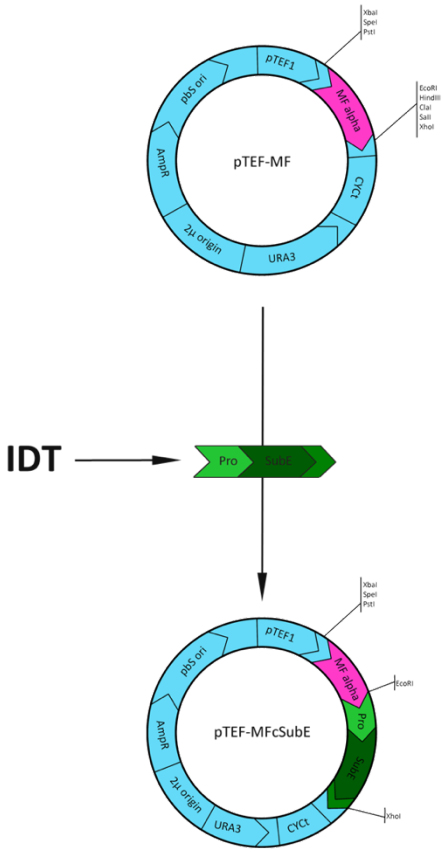

Figure 1: Cloning of codon optimized Subtilisin E into pTEF-MF

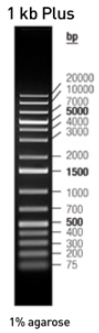

Figure 2: 1kb Thermo scientific ladder

For all agarose gels we used 1% agarose and 1 kb Thermo scientific ladder 08:

Day 1

Codon Optimization

We used the IDT codon optimization tool to convert the *E. coli* optimized Subtilisin E gene into one that *S. cerevisiae* can translate better.

The sequence of the codon optimized construct can be seen as part BBa_K2020023.

Day 7

Dilution

After the gBlock from IDT arrived we diluted the it as described in their instructions.

PCR Amplification of gBlock

Just restricting the gBlock and then clone it into a vector does not work without prior amplification. That's why we first amplified it with the primers YP0028 and YP0029 according to the PCR protocol.

Cycles:

ID	92°C	1 min	
D	92°C	20 sec	X24
A	60-70°C	30 sec	
E	72°C	50 sec	
FE	72°C	3 min	
S	4°C	storage	

Agarose Gel

We prepared an agarose gel to see if the PCR product is present and has the right size of about 1100 bp.

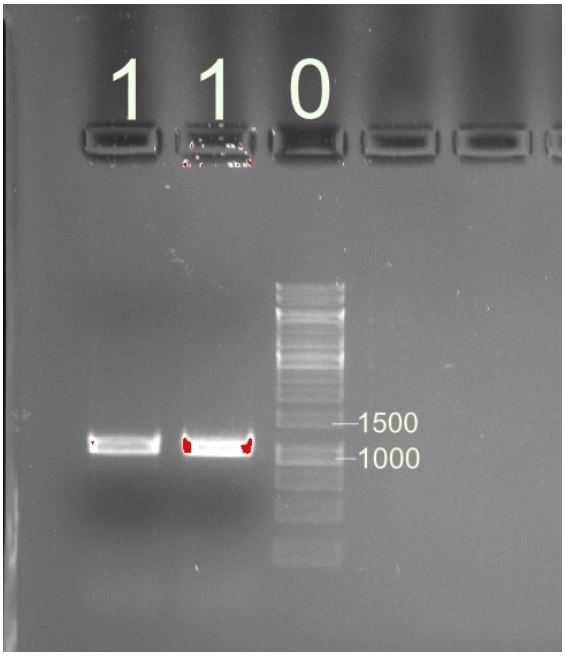

Figure 3; Agarose gel; 1: PCR product after amplification; 0: ladder

In both samples PCR product is visible on agarose gel at the right size.

PCR Clean Up
We did a PCR clean-up of the amplified codon optimized Subtilisin E.

Nanodrop
We did a nanodrop of the cleaned up samples of codon optimized Subtilisin E to determine DNA concentration.

1. 244.7 ng/µl
2. 220.9 ng/µl

Restriction
We double digested the codon optimized Subtilisin E and the pTEF-MF backbone with EcoRI and XhoI.

Dephosphorylation
We dephosphorylated the double digested backbone pTEF-MF.

Ligation
The amplified codon optimized Subtilisin E was ligated with the dephosphorylated pTEF-MF backbone and as control we made a self-ligation of the dephosphorylated backbone

Pipetting scheme:

1. 1:9 ratio regarding the concentration in ng/µl
2. 1:9 ratio regarding DNA molecules

We ligated half of each sample for 3h at 16°C and the other half overnight in case this was not efficient enough.

Transformation in *E. coli* Dh5 alpha
We transformed 2 and 5 µl of the 3 h ligation into DH5alpha respectively.

Day 9

Transformation Results
The transformation was successful, but we only had one colony.

Colony PCR
We made a colony PCR of the picked clone with primers YP0016 and YP0017. As the ideal annealing temperature was not yet known, we used a gradient with 11 different temperatures.

Cycles:

ID	98°C	30 sec
D	98°C	10 sec
A	58-65°C	30 sec
E	72°C	90 sec
FE	72°C	10 sec
S	4°C	storage

Agarose Gel

After performing the colony PCR, we loaded the samples on an agarose gel. The expected size was about 1400 bp.

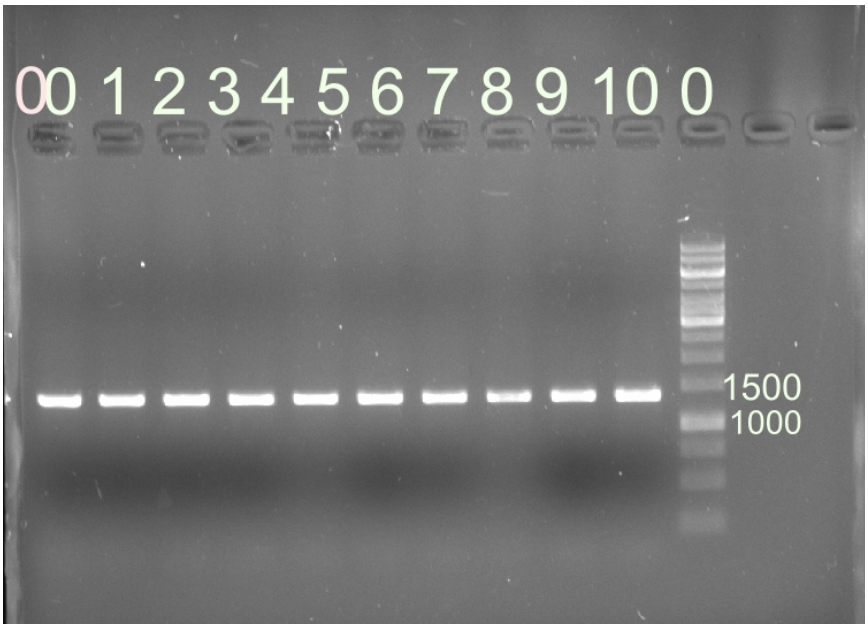

Figure 4; Agarose gel; 00: PCR product of colony 00; 1: PCR product of colony 1; 2: PCR product of colony 2; 3: PCR product of colony 3; 4: PCR product of colony 4; 5: PCR product of colony 5; 6: PCR product of colony 6; 7: PCR product of colony 7; 8: PCR product of colony 8; 9: PCR product of colony 9; 10: PCR product of colony 10

It is visible that every temperature created PCR product at the right size and all temperatures worked for our primers.

Day 10

Plasmid Isolation

We isolated the plasmid of the overnight pTEF-MF with codon optimized Subtilisin E we made during the colony PCR.

Sequencing

We sent the isolated plasmid in for sequencing with primers YP0019 and YP0030.

Day 11

Sequencing Results

The sequencing results of clone 2 were perfect.

Transformation in Yeast

We transformed the clone 2 of pTEF-MF with codon optimized Subtilisin E into CENPK2-1D.

Results

After day 12 we had the expression system with confirmed sequence our host organism.
After the transformation in CENPK2-1D we tested the expression of Subtilisin E.